Effective targeting of the survivin dimerization interface with small molecule inhibitors

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Abstract

Many oncoproteins are considered undruggable because they lack enzymatic activities. In this study, we present a small molecule-based anticancer agent that acts by inhibiting dimerization of the oncoprotein survivin, thereby promoting its degradation along with spontaneous apoptosis in cancer cells. Through a combination of computational analysis of the dimerization interface and in-silico screening, we identified one compound that induced proteasome-dependent survivin degradation. Analysis of a set of structural analogues led us to identify a lead compound (LQZ-7F) which was effective in blocking the survival of multiple cancer cell lines in a low micromolar concentration range. LQZ-7F induced proteasome-dependent survivin degradation, mitotic arrest, apoptosis, and it blocked the growth of human tumors in mouse xenograft assays. In addition to providing preclinical proof of concept for a survivin-targeting anticancer agent, our work offers novel in silico screening strategies to therapeutically target homo-dimeric oncogenic proteins considered undruggable.

Introduction

Survivin is a member of the Inhibitor of apoptosis (IAP) gene family containing a single Baculovirus IAP Repeat (BIR) domain, a zinc-finger fold, and an extended C-terminal helical coiled coil (1). It is a homo-dimer of a 16.5-kDa protein (2,3). Ectopic survivin over-expression causes inhibition of cell death induced by intrinsic and extrinsic stimuli in cell lines (4-9) and in animals (10). Survivin is over-expressed essentially in all cancers, but not expressed in most adult normal tissues (1,11). Survivin has also been shown to contribute to radiotherapy and chemotherapy resistance, and inhibition of survivin sensitizes cancer cells to these treatments (12-14). Treatments with molecular probes such as antisense oligonucleotide, ribozyme, siRNA, and dominant negative mutant all resulted in caspase-dependent cell death and increased apoptosis induced by radiation and anticancer drugs (6,14-19). These findings clearly established survivin as an ideal target for discovery of anticancer therapeutics.

Unfortunately, survivin belongs to a group of proteins that are considered undruggable due to lack of enzymatic activities. Although small molecule inhibitors have been identified that would interfere with the function of this type of proteins by blocking their interaction with other essential proteins, this approach has not led to drug candidates for clinical trials except in rare cases. In the case of survivin, targeting its expression has been attempted to avoid targeting survivin protein directly. For example, YM155, a small molecule compound, has been identified to inhibit survivin expression by targeting its transcription (20) and later was shown to inhibit transcription factors such as SP1 (21). However, several phase II trials of YM155 showed only limited or modest at best efficacy on human cancers (21). An antisense oligonucleotide, LY2181308, that inhibits survivin expression has also been tested as a single agent in phase I trial for solid tumors (22) and in combination with docetaxel in phase II trial for castration-resistant prostate cancers (23).

Unfortunately, neither of these two trials showed any benefit of using LY2181308. Thus, new inhibitors of survivin are clearly needed perhaps by developing a strategy to target directly at the survivin protein itself.

It is known that exposure of the hydrophobic interface of a dimeric protein often leads to conformational change (24,25), which causes destabilization and degradation of the protein by proteasome or autophagy (26). Because survivin exists as a homo-dimer, we hypothesized that a small molecule compound that inhibits survivin dimerization may promote survivin degradation via the proteasome and, thus, eliminates the protein and leads to spontaneous apoptosis. We recently developed a novel strategy to identify interfacial hydrophobic core units critical for homo-dimerization (27,28). Using this strategy, we tested the above hypothesis by first identifying the hydrophobic core residues critical for survivin dimerization followed by in-silico screening for inhibitors targeting the critical core residues as well as in-vitro and cell-based assays. We identified a hit compound, LQZ-7, which dissociated dimeric survivin and induced proteasome-dependent survivin degradation. Further analysis helped identify several active analogues of the hit compound, which resulted in a potential lead compound (LQZ-7F) that inhibits survival of multiple cancer cell lines with low micromolar IC_{50}, suppresses xenograft tumor growth, and inhibits survivin in vivo.

Materials and Methods

Materials and cell lines. Antibodies against FLAG tag (F3165), α-tubulin (T9026), and β-actin (A5316) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibody against survivin (2808), iScriptTM cDNA Synthesis Kit, StepOnePlus™ Systems kit, and the Metafectene Pro transfection reagent were purchased from Cell Signaling Technology (Danvers, MA, USA), Bio-Rad Laboratories (Hercules, CA, USA), Applied Biosystems (Warrington, UK), Biontex

(München, Germany), respectively. The enhanced chemiluminescence reagents and CNBr-activated Sepharose 4B were from GE Healthcare (Uppsala, Sweden). Cell culture media and fetal bovine serum were from Media Tech (Herndon, CA) or Applied Biosystems-Life Technologies (Carlsbad, CA, USA). All other chemicals were purchased from Sigma or Fisher Scientific. The human cancer cell lines used in this study were all from ATCC and have been authenticated by short tandem repeat analysis on January 21, 2013.

MD Simulation analysis of water trafficking. MD simulations of survivin dimers and water trafficking were carried out using the AMBER9 package as previously described (27). Crystal structure of survivin dimer with PDB code 1F3H was acquired from RCSB protein databank (2). Zinc parameters were developed by Y.P. Pang using the cationic dummy atom (cada) approach (29). Survivin dimer was solvated in a truncated octahedron box with edges no closer than 10Å to any atom in the solute with appropriate number of counter ions added to neutralize each system. Particle Mesh Ewald (PME) was used to calculate the long-range electrostatic interactions and the nonbonded cutoff was set to 8.0 Å. Each system was equilibrated by a four-step protocol prior to the 20-ns production MD simulation.

Total buried dimeric interface areas and buried surface areas decomposed to each residue were calculated by areaimol of the CCP4 package. Dimerization core residues that have more than 75% solvent accessible area buried in the dimeric interface were selected. Survivin has one dimerization core unit consisting of four residues: Leu^{98} and Phe^{101} from one chain and same residues from another chain. This dimerization core entity is further validated by its water exchange rate determined via 20-ns water explicit MD simulation as previously described (27,28). A sphere of 6 Å in radius was drawn from the center in the mass of the core. Water molecules that fell into the sphere during the simulation were monitored and their residue IDs were recorded by

VMD program. Then, water molecules from every 20 frames (representing a 200 ps timespan) were pooled and compared with that from the previous 20 frames. Water molecules with residue IDs that existed in the current 20 frames but did not appear in the previous 20 frames were considered water molecules that moved in during the current 200 ps timespan. Water molecules with residue IDs found in the current 20 frames but did not show up again in the next 20 frames were considered water molecules that moved out during this 200 ps timespan.

In-Silico Virtual Screening. Structure-based in-silico screening was performed as previously described (30,31). Briefly, the 3-D coordinates of survivin were acquired from PDB code 1F3H. Only one of the two chains was kept and the protein chain was prepared for docking. Molecular surface was calculated using DMS (Distributed Molecular Surface) program. Partial charges and protons were added to the protein by UCSF Chimera Dock Prep module (32). In-silico dock screening of 200,000 compounds from SPECS's library (www.specs.net) was performed using UCSF DOCK 6.0 program (33). The docking of each compound was first scored with the DOCK GRID scoring function (34). The top-scoring 1000 compounds were analyzed again and re-scored using the AMBER scoring function of DOCK 6.0 package (35). These compounds were clustered using MOE (Molecular Operating Environment) program and visually examined using the UCSF Chimera ViewDock function. Final 100 compounds were selected based on the combination of GRID and AMBER score, drug likeness (Lipinski's rule of five), and on consideration of maximizing compounds from different clusters.

Non-denaturing PAGE. 1-μg purified survivin were incubated with 20 μM candidate compounds, DMSO vehicle control, or different concentrations of LQZ-7 before mixing with equal volume of 2 sample buffer (100 mM Tris, pH 8.0, 20% glycerol, 0.005% bromophenol blue, 2% Triton X-100, and 100 mM DTT) followed by incubation at room temperature for 30 min.

After centrifugation at 11,000 × g for 10 minutes, the supernatants were separated by electrophoresis on 15% Tris/glycine polyacrylamide gel followed by transfer to PVDF membrane for western blot analysis as previously described (36).

Survival assays (MTT and colony formation). These assays were performed as previously described (37,38). Briefly, MTT assay was performed by seeding 2500 cells in 96-well plate and cultured 24 hours before addition of survivin inhibitors at different concentrations and continuous culture for 3 days. The cells were then subjected to MTT assay. For colony formation assay, 100 cells/well were seeded in 6-well plates and cultured for 24 hours before addition of survivin inhibitors or DMSO vehicle. The cells were continuously cultured in the presence of survivin inhibitors or DMSO for 10-14 days followed by staining with crystal violet and counting manually.

Fluorogenic assay. Fluorogenic assay was performed as previously described (39). Briefly, LQZ-7 was pre-incubated without or with 10 μg purified survivin for 30 min at 37ºC followed by determination of fluorescence emission at 485 nm with excitation at 590 nm. A dose response-curve was fitted to an equation describing one-site binding model to determine the K_d of LQZ-7 binding to survivin using GraphPad Prism 4.0 software.

Apoptosis assay. Annexin V apoptosis assay was performed using a kit as described by the manufacturer (Invitrogen). Briefly, treated cells were harvested after washing with PBS and then resuspended in annexin-binding buffer (10mM HEPES, pH7.4, 120 mM NaCl, 2.5mM $CaCl_2$) at the density of 1×10^6 cells/ml. After addition of Alexa Fluor 488 annexin V and propidium iodide, the cells were incubated for 15 minutes at room temperature followed by dilution with annexin-binding buffer and FACS analysis of fluorescence emission at 530 nm and 575 nm with 488 nm excitation.

Half-life determination and effect of proteasome inhibitors on survivin degradation. The effect of survivin inhibitors on the half-life of survivin was determined as previously described (40). Briefly, PC-3 or DU145 cells were pre-treated with 2 μM cycloheximide for 1 hour followed by incubation without or with 9 μM LQZ-7 or 5 μM LQZ-7F for different times. The cells were then harvested for Western blot analysis of survivin.

For the proteasome inhibitor rescue experiment, PC-3 cells were seeded in 10-cm dishes at 8×10^5 cells/dish and cultured for 48 hours followed by replacement with fresh media containing DMSO control, 7 μM MG-132 or 70 nM bortezomib and incubation for 2 hours. LQZ-7 and LQZ-7F were then added to the culture to final concentrations of 10 and 5 μM, respectively, and incubated for additional 24 hours. The cells were then harvested and washed with PBS and lysed for Western blot analysis of survivin.

Efficacy analysis in xenograft mouse model. For efficacy study, 3×10^6 PC-3 cells were injected subcutaneously in the flanks of 10 4-6 week old male NOD/SCID mice. When the tumor volume reached ~100 mm^3, the mice were randomized into two different groups (5/group) with one group treated by vehicle control and the other by LQZ-7F at 25 mg/kg via IP injection once every three days for total of 8 treatments. Tumor volume and body weight were measured every two days. On the 30^{th} day after the initial treatment, mice were euthanized and the tumor tissues were harvested, weighed, and subjected to hematoxylin and eosin (H&E) staining as well as Western blot and immunohistochemistry analysis of survivin.

Results

Analysis of dimerization domain of survivin. To effectively target the dimerization domain of survivin, we first analyzed the dimerization interface to identify residues that are critical for survivin dimerization as targets for in-silico screening. The interacting residues between the two

identical subunits are comprised of residues 6-10, 93-99 and 101-102 (Leu^{6}, Pro^{7}, Pro^{8}, Ala^{9}, Trp^{10}, Phe^{93}, Glu^{94}, Glu^{95}, Leu^{96}, Thr^{97}, Leu^{98}, Gly^{99}, Phe^{101}, Leu^{102}). About 80% of these residues are hydrophobic, which is comparably high considering the average of non-polar interaction is only ~50% in dimeric proteins [1]. The dimeric survivin has a total calculated solvent accessible area of 18,039 $Å^2$. The buried accessible area in the dimeric interface of a monomeric survivin is 550 $Å^2$ and it occupies only 6% of the total accessible area of a monomer (9,044 $Å^2$), which is much less than the average value (~20%) in dimeric or oligomeric proteins [1,2,3]. The relatively small area and the high hydrophobicity of the interactive surface indicate that it may be a good target site for drug discovery.

Previously, we found that dimeric proteins may have dimerization core units that are sealed from water penetration and are critical for homo-dimerization (27). Further analysis of the dimeric interface using our previously described computational approach (27) shows that survivin has one dimerization core unit consisting of four residues, Leu^{98} and Phe^{101} from one subunit and the same residues from another subunit (Fig. 1A). Water exchange rate in this dimerization core unit was determined by performing 20-ns water explicit MD simulation and a computational method developed by us previously (27). As shown in Fig. 1B, few water molecules moved in or out of the dimerization core during the 20-ns simulation with an estimated water exchange rate of 0.5 water/200 ps, which is comparable with that of the 14-3-3σ dimerization core (27). It is, however, significantly lower than that of the mutant 14-3-3σ molecule, which lost dimerization activity with a much higher water exchange rate (>4 water/200 ps). These findings suggest that the dimerization core unit of survivin consisting of Leu^{98} and Phe^{101} is tightly sealed and may be critical for the formation of stable survivin dimers. Disrupting this core formation may affect survivin

dimerization. Indeed, mutation of Phe^{101} to Ala^{101} together with Leu^{102} to Ala^{102} mutation has been shown to disrupt survivin dimerization (41).

Identification of LQZ-7 targeting the dimerization domain of survivin. To identify small molecule compounds that can potentially inhibit survivin dimerization, we performed in-silico screening of ~200,000 compounds targeting the critical hydrophobic core residues Leu^{98} and Phe^{101} in the dimeric interface using DOCK. Of the 100 top-scoring compounds of diversified structures, 49 chemical samples were commercially available and tested first for their cytotoxicity using two human cancer cell lines DU145 and PC-3. As shown in supplemental Fig. S1, only compounds 4, 7, 9, 12, 21, 36, and 42 at 20 μM were able to inhibit ≥50% survival of both DU145 and PC-3 cells. Consequently, these compounds were chosen for further investigation.

The selected compounds were tested for their ability to dissociate survivin dimers using purified survivin and non-denaturing PAGE analysis. As shown in Fig. 2A, compound #7 (named LQZ-7 with structure shown in Fig. 2B) was able to affect the mobility of purified survivin, presumably by dissociating the dimeric survivin into monomers. To confirm its activity and test its potential selectivity, we had LQZ-7 resynthesized and performed a dose-response analysis of its activity in dissociating dimeric survivin and an irrelevant control but similar dimeric protein, 14-3-3σ. Although 100 μM LQZ-7 appears to be required to completely dissociate dimeric survivin (Fig. 2C), LQZ-7 at 100 μM had no effect on 14-3-3σ dimerization (Fig. 2D). We also performed non-denaturing PAGE analysis of nascent proteins synthesized in a cell-free system in the presence of LQZ-7. As shown in Fig. 2E, 20 μM LQZ-7 achieved a complete inhibition of survivin dimerization. Together, these findings suggest that LQZ-7F can inhibit dimerization of nascent survivin and dissociate existing dimeric survivin with selectivity over other homo-dimeric proteins such as 14-3-3σ. The finding that less LQZ-7 is required to completely inhibit

dimerization of nascent survivin than to completely dissociate existing dimeric survivin suggests that inhibiting dimerization of newly synthesized proteins may be dynamically favorable than dissociating the existing dimeric proteins.

To verify that LQZ-7 indeed binds to survivin and inhibits survivin dimerization, we took advantage of the intrinsic fluorescent property of LQZ-7 and performed a fluorogenic titration assay in the presence or absence of survivin as previously described (39). Fig. 2F shows that the intrinsic fluorescence of LQZ-7 dramatically increases in the presence of recombinant survivin, indicating that LQZ-7 likely interacts with survivin. The K_d of LQZ-7 binding to survivin is estimated to be ~0.24±0.11 μM.

LQZ-7 accelerates proteasome-dependent degradation of survivin. Exposure of hydrophobic interface of a dimeric protein often leads to conformational change (24,25), which causes destabilization and degradation of the protein by proteasome or autophagy (26). We, thus, hypothesized that LQZ-7 may cause proteasome-dependent degradation of survivin by dissociating survivin dimers and exposing the hydrophobic dimeric interface. To test this hypothesis, we first treated DU145 cells with LQZ-7 for different times followed by determination of endogenous survivin using Western blot analysis. Fig. 3A shows that LQZ-7 treatment indeed reduces survivin protein level compared with vehicle control treatment. Similar results were also observed with PC-3 cells (data not shown). However, LQZ-7 treatment had no effect on the level of survivin mRNA as determined using real time RT-PCR (Fig. 3B). LQZ-7 treatment also effectively reduced the level of ectopic Flag-tagged survivin in HEK293 cells under the control of an exogenous CMV promoter (Fig. 3C). These observations together suggest that survivin loss induced by LQZ-7 is likely at the protein but not mRNA level.

We next determined if LQZ-7 causes survivin degradation by examining its effect on survivin half-life. For this purpose, DU145 and PC-3 cells were pre-treated with cycloheximide to inhibit synthesis of new proteins followed by treatment with LQZ-7 for different times. Fig. 3D-E show that the half-life of survivin is 1.5-2.5 hrs in control-treated DU145 and PC-3 cells, consistent with previously reported survivin half-life (42,43). However, following LQZ-7 treatment the half-life of survivin was reduced to ~30 min. Co-treatment with a proteasome inhibitor, MG132 or bortezomib, reversed LQZ-7-induced survivin loss (Fig. 3F), consistent with a previous report that survivin degradation is mediated by the proteasome (44). We also found that LQZ-7 does not inhibit survivin protein synthesis as determined using [^{35}S]methionine pulse-labeling in combination with immunoprecipitation of survivin following LQZ-7 treatment (data not shown). Together, the above findings suggest that LQZ-7 may cause proteasome-dependent survivin degradation possibly by inhibiting dimerization and exposing the hydrophobic core residues of survivin.

*Characterization of LQZ-7 analogues***.** Although LQZ-7 dissociates survivin dimer in vitro and causes survivin degradation in cells, cell-based cytotoxicity assays showed that the IC_{50} of LQZ-7 in human PC-3 and DU145 cells was ~25 μM (Fig. 4B). The modest IC_{50} may be due to the possibility that the carboxyl group in LQZ-7 (Fig. 4A) impedes its cellular permeability. To improve cellular effect of LQZ-7, we searched the SPECS database and identified six commercially available analogues (LQZ-7A, B, C, D, E, and F, Fig. 4A). The chemical samples of these analogues were obtained and tested first for their cytotoxicity to DU145 and PC-3 cells compared with the initial hit LQZ-7. Fig. 4B shows that 5 of the 6 analogues have much lower IC_{50} than LQZ-7 for both cells. These analogues together with LQZ-7 were then used to test their effect on the expression of ectopic Flag-tagged survivin in HEK293 cells. As shown in Fig. 4C, while

LQZ-7A, D, and E had no effect on the level of Flag-tagged survivin, LQZ-7B, C, and F all reduced survivin protein level. It appears that LQZ-7C and F completely eliminated survivin whereas the parent compound LQZ-7 did not, consistent with their lower IC_{50} than the parent compound. The fact that LQZ-7E has high IC_{50} and does not reduce survivin protein suggests that LQZ-7E may not bind to and inhibit survivin. It remains unknown why LQZ-7A and D have no effect on survivin level while maintaining low IC_{50} (see Discussion).

Of the two analogous (LQZ-7C and F) that have the best IC_{50} and ability to eliminate survivin, LQZ-7F is unique, smaller, and simpler in structure with a primary amine group as an advantage for further study (see below). Thus, we elected to pursue LQZ-7F further as a potential lead and tested its activity in suppressing the expression and inducing degradation of endogenous survivin. Similar as LQZ-7, LQZ-7F effectively suppressed endogenous survivin expression in both DU145 and PC-3 cells (Fig. 4D) and increased degradation of endogenous survivin (Fig. 4E). Furthermore, proteasome inhibitors MG132 and bortezomib both were able to rescue LQZ-7F-induced survivin degradation (Fig. 4F). Thus, LQZ-7F, similar as its parent compound LQZ-7, also induces survivin degradation in a proteasome-dependent manner.

LQZ-7F interaction with survivin. To investigate if LQZ-7F indeed binds to survivin, we took advantage of the primary amine group and immobilized LQZ-7F onto CNBr-activated Sepharose for a pull-down assay using purified survivin. As shown in Fig. 4G, survivin was successfully pulled down by Sepharose-immobilized LQZ-7F, but not by the control beads without LQZ-7F. Thus, LQZ-7F, similar to LQZ-7, may bind directly to survivin. To understand how LQZ-7F interacts with survivin, we performed docking analysis of LQZ-7F in the dimerization interface of survivin, which revealed two key interactions between LQZ-7F and survivin: (a) H-bond between the primary amine group of LQZ-7F and Glu^{94} of survivin; (b) π-π stacking and hydrophobic

interaction between the tetracyclic furazanopyrazine ring of LQZ-7F and the hydrophobic residues Trp^{10} and Phe^{93} in addition to the hydrophobic core residues Leu^{98} and Phe^{101} (Fig. 4H).

LQZ-7F inhibits survival of multiple cancer cell lines by inducing apoptosis. Survivin is ubiquitously up-regulated in human cancers and, thus, inhibiting survivin may be a general approach to help eradicate many cancers. Toward this goal, we tested the effect of LQZ-7F on survival of multiple human cancer cell lines representing acute myeloid leukemia and cancers of breast, colon, lung, pancreas, prostate, and ovary using MTT assay. As shown in Fig. 5A, LQZ-7F effectively inhibited survival of all cancer cell lines with IC_{50} ranging between 0.4-4.4 μM.

The activity of LQZ-7F in suppressing cancer cell survival was further evaluated using colony formation assay for PC-3 and A549 cells. As shown in Fig. 5B, the colony formation efficiency of PC-3 cells was reduced from ~62% of the control to ~52, 12, and 8% following treatments with LQZ-7F at 0.2, 0.5, and 1 μM, respectively. A549 cells were more sensitive to LQZ-7F with lower IC_{50} than PC-3 cells and the colony formation efficiency were consistently reduced more from 45% of the control treatment to ~2.5% and 0% treated by 0.2 and 0.5 μM of LQZ-7F, respectively.

Previously, it has been shown that dominant negative survivin causes spontaneous apoptosis of PC-3, DU145, and LNCaP cells (12). We next tested if LQZ-7F also causes spontaneous apoptosis by inhibiting survivin as determined by Annexin V staining. For this experiment, we tested PC-3 and HL-60 as representative cells because PC-3 has high while HL-60 has intermediate IC50 against LQZ-7F (Fig. 5A). As shown in Fig. 5C, 54-69% apoptosis for PC-3 and 66-98% apoptosis for HL60 cells were generated following treatments with 5-10 μM LQZ-7F. These findings were further validated by determining the cleavage of PARP-1, a substrate of caspases during execution of apoptosis, in both PC-3 and HL60 cells following LQZ-7F

treatments (Fig. 5D).

Finally, we tested survivin expression in all 13 cell lines (Fig. 5E) and performed a correlation analysis between survivin level and IC_{50}. As shown in Fig. 5F, the IC_{50} values strongly associate with survivin protein level in these cells with a Pearson correlation coefficient of 0.52, indicating that LQZ-7F may suppress the survival of these cancer cells by acting on survivin.

LQZ-7F treatments disrupt microtubule structure and cause mitotic arrest. Survivin has been shown to have dual functions in inhibiting apoptosis and in promoting cell cycle progression (1). The later was thought to derive from survivin action in destabilizing microtubules both in vitro and in vivo (45,46). Thus, to further determine LQZ-7F effect on survivin, we analyzed microtubule structure following LQZ-7F treatment in PC-3 cells. As shown in Fig. 6, the control DMSO-treated cells have orderly microtubule fibers in all cells. However, the microtubule structure was severely disrupted following LQZ-7F treatments. It also appears that many cells are arrested in mitotic phase with aberrant spindles following LQZ-7F treatments. These observations are consistent with the role of survivin in microtubule dynamics (47,48) and in proper chromosome segregation and cytokinesis (49,50).

LQZ-7F inhibits growth of xenograft tumors by inhibiting survivin. We next determined if LQZ-7F is active in suppressing tumor growth in vivo using a xenograft animal model. For this purpose, NOD/SCID mice were first implanted subcutaneously with PC-3 cells to establish xenograft tumors. PC-3 was chosen because it has the highest IC_{50} (Fig. 5). When the xenograft tumor reached the size of ~100 mm^3, the mice were randomized into two groups and treated with 25 mg/kg LQZ-7F or vehicle control via IP injection once every three days for a total of 8 treatments. As shown in Fig. 7A, the growth of xenograft tumors was significantly inhibited in the LQZ-7F-treated group compared to the tumors in vehicle control-treated group. However, the body

weight of mice in the treatment group remained constant after multiple dosing (Fig. 7B), indicating that LQZ-7F may not cause major toxicity after multiple dosing. In fact, the body weight of the control group dropped slightly possibly due to disease burden of xenograft tumors. This symptom appears to be alleviated by LQZ-7F treatment, consistent with the smaller tumor size in the treatment group.

The final dissected tumors in the treatment group appear to be small and round with smooth surface whereas the tumors of the control group appear to be irregular and bigger (Fig. 7C), suggesting that the tumors in the treatment group are confined whereas the tumors in the control group are aggressive. The average weight of the tumors in the treatment group is significantly less than that in the control group (Fig. 7D). The LQZ-7F-treated tumors also appear to have more apoptotic cells as indicated by condensed chromatin than the control-treated tumors (Fig. 7E). Western blot and IHC staining analyses showed that survivin in xenograft tumors of the LQZ-7F-treated group was dramatically reduced compared with the tumors of the control-treated group (Fig. 7E-F), suggesting that the effect of LQZ-7F on xenograft tumor growth may be due to its binding to survivin and induction of survivin degradation in vivo.

Discussion

In this study, using in-silico screening targeting the critical hydrophobic core residues in the dimeric interface following detailed analysis of the buried surface area, we successfully identified a hit compound (LQZ-7) and a potential lead inhibitor (LQZ-7F) that can bind directly to survivin and cause proteasome-dependent survivin degradation. LQZ-7F has an IC_{50} of 0.4-4.4 μM against multiple cell lines of different human cancers and induces spontaneous apoptosis. It is also effective in suppressing xenograft tumor growth and reduces survivin level in xenograft tumors.

This study provides a proof-of-concept that the hydrophobic core residues in the dimeric interface of undruggable homo-dimeric proteins can be targeted for drug discovery. Combining computational analysis of the dimeric interface to first identify these core units with in-silico screening, as demonstrated here, is likely a viable approach that will help succeed in identifying small molecule compounds inhibiting dimerization of the target protein.

The finding that LQZ-7 and its analogues induce survivin degradation is consistent with the concept that exposure of the hydrophobic interface of a dimeric protein often lead to conformational change (24,25), which causes destabilization and degradation of the protein by the proteasome or autophagy (26). Interestingly, not all LQZ-7 analogues induced survivin degradation. LQZ-7E had no effect on survivin expression, consistent with its lack of inhibitory effect on cell survival, suggesting that LQZ-7E may not bind to and induce survivin degradation. However, compounds LQZ-7A and 7D are effective in suppressing cancer cell growth without inducing survivin degradation. This finding is intriguing and suggests that these two compounds may bind to survivin and inhibit its function but do not trigger proteasome-dependent survivin degradation. Alternatively, these compounds may have off-target effects that inhibit cell survival but lost their effect on survivin protein. Clearly, future studies are needed to test these possibilities.

It is noteworthy that mutation of the hydrophobic core residue Phe^{101} to Ala^{101} together with mutation of Leu^{102} to Ala^{102} disrupted survivin dimerization, which did not appear to result in survivin degradation (41). This observation apparently is different from our finding using small molecule inhibitors to disrupt survivin dimerization. Although the cause for this difference is currently unknown, it is possible that the mutant proteins do not exist as true monomers in mammalian cells because they can form heterodimers by binding to other proteins such as CRM1 (41). On the other hand, the monomers induced by small molecule inhibitor such as LQZ-7F may

not form heterodimer with other proteins due to existence of the compound in the interface. It is also possible that mutation of the hydrophobic core residues reduced the hydrophobicity of the interface and, thus, the protein could escape from the cell quality control system while binding of the small molecule compound to the interface may increase the hydrophobicity and attract the quality control system. Future studies are warranted to test these possibilities.

It is also noteworthy that a small molecule compound, LLP3, was synthesized previously based on the Abbot8, a survivin inhibitor obtained via a NMR-based screening. LLP3 was thought to bind to the dimerization interface of survivin (39). However, LLP3 had no effect on survivin dimerization or its expression. Instead, it inhibited interaction between survivin and its partner Ran protein. Thus, it is not clear if LLP3 truly binds to the dimerization interface of survivin. Nevertheless, LLP3 inhibited proliferation of cancer cells but with a much higher IC_{50} of 14-38 μM.

References

1. Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003;22(53):8581-9.
2. Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat Struct Biol 2000;7(7):602-8.
3. Chantalat L, Skoufias DA, Kleman JP, Jung B, Dideberg O, Margolis RL. Crystal structure of human survivin reveals a bow tie-shaped dimer with two unusual alpha-helical extensions. Mol Cell 2000;6(1):183-9.
4. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998;58(23):5315-20.
5. Suzuki A, Ito T, Kawano H, Hayashida M, Hayasaki Y, Tsutomi Y, et al. Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. Oncogene 2000;19(10):1346-53.
6. Kasof GM, Gomes BC. Livin, a novel inhibitor of apoptosis protein family member. J Biol Chem 2001;276(5):3238-46.
7. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem 2002;277(5):3247-57.
8. Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, et al. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene 2002;21(17):2613-22.
9. Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, et al. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. Cell Mol Life Sci 2002;59(8):1406-12.
10. Grossman D, Kim PJ, Blanc-Brude OP, Brash DE, Tognin S, Marchisio PC, et al. Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. J Clin Invest 2001;108(7):991-9.
11. Fukuda S, Pelus LM. Survivin, a cancer target with an emerging role in normal adult tissues. Mol Cancer Ther 2006;5(5):1087-98.
12. Zhang M, Mukherjee N, Bermudez RS, Latham DE, Delaney MA, Zietman AL, et al. Adenovirus-mediated inhibition of survivin expression sensitizes human prostate cancer cells to paclitaxel in vitro and in vivo. Prostate 2005;64(3):293-302.
13. Zhang M, Latham DE, Delaney MA, Chakravarti A. Survivin mediates resistance to antiandrogen therapy in prostate cancer. Oncogene 2005;24(15):2474-82.
14. Pennati M, Colella G, Folini M, Citti L, Daidone MG, Zaffaroni N. Ribozyme-mediated attenuation of survivin expression sensitizes human melanoma cells to cisplatin-induced apoptosis. J Clin Invest 2002;109(2):285-6.
15. Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol 1999;1(8):461-6.
16. Chen J, Wu W, Tahir SK, Kroeger PE, Rosenberg SH, Cowsert LM, et al. Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. Neoplasia 2000;2(3):235-41.

17. Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60(11):2805-9.
18. Williams NS, Gaynor RB, Scoggin S, Verma U, Gokaslan T, Simmang C, et al. Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. Clin Cancer Res 2003;9(3):931-46.
19. Choi KS, Lee TH, Jung MH. Ribozyme-mediated cleavage of the human survivin mRNA and inhibition of antiapoptotic function of survivin in MCF-7 cells. Cancer Gene Ther 2003;10(2):87-95.
20. Nakahara T, Takeuchi M, Kinoyama I, Minematsu T, Shirasuna K, Matsuhisa A, et al. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. Cancer Res 2007;67(17):8014-21.
21. Rauch A, Hennig D, Schafer C, Wirth M, Marx C, Heinzel T, et al. Survivin and YM155: How faithful is the liaison? Biochim Biophys Acta 2014;1845(2):202-20.
22. Tanioka M, Nokihara H, Yamamoto N, Yamada Y, Yamada K, Goto Y, et al. Phase I study of LY2181308, an antisense oligonucleotide against survivin, in patients with advanced solid tumors. Cancer Chemother Pharmacol 2011;68(2):505-11.
23. Wiechno P, Somer BG, Mellado B, Chlosta PL, Cervera Grau JM, Castellano D, et al. A randomised phase 2 study combining LY2181308 sodium (survivin antisense oligonucleotide) with first-line docetaxel/prednisone in patients with castration-resistant prostate cancer. Eur Urol 2014;65(3):516-20.
24. Agashe VR, Shastry MC, Udgaonkar JB. Initial hydrophobic collapse in the folding of barstar. Nature 1995;377(6551):754-7.
25. Lins L, Brasseur R. The hydrophobic effect in protein folding. FASEB J 1995;9(7):535-40.
26. Kubota H. Quality control against misfolded proteins in the cytosol: a network for cell survival. J Biochem 2009;146(5):609-16.
27. Liu JY, Li Z, Li H, Zhang JT. Critical residue that promotes protein dimerization: a story of partially exposed phe(25) in 14-3-3sigma. J Chem Inf Model 2011;51(10):2612-25.
28. Li Z, Peng H, Qin L, Qi J, Zuo X, Liu JY, et al. Determinants of 14-3-3sigma protein dimerization and function in drug and radiation resistance. J Biol Chem 2013;288(44):31447-57.
29. Pang YP. Successful molecular dynamics simulation of two zinc complexes bridged by a hydroxide in phosphotriesterase using the cationic dummy atom method. Proteins 2001;45(3):183-9.
30. Neher TM, Shuck SC, Liu JY, Zhang JT, Turchi JJ. Identification of novel small molecule inhibitors of the XPA protein using in silico based screening. ACS Chem Biol 2010;5(10):953-65.
31. Huang W, Dong Z, Wang F, Peng H, Liu JY, Zhang JT. A Small Molecule Compound Targeting STAT3 DNA-Binding Domain Inhibits Cancer Cell Proliferation, Migration, and Invasion. ACS Chem Biol 2014;9(5):1188-96.
32. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem 2004;25(13):1605-12.
33. Lang PT, Brozell SR, Mukherjee S, Pettersen EF, Meng EC, Thomas V, et al. DOCK 6: combining techniques to model RNA-small molecule complexes. RNA 2009;15(6):1219-30.

34. Meng EC, Shoichet BK, Kuntz ID. Automated Docking with Grid-Based Energy Evaluation. J Comput Chem 1992;13(4):505-24.
35. Graves AP, Shivakumar DM, Boyce SE, Jacobson MP, Case DA, Shoichet BK. Rescoring docking hit lists for model cavity sites: predictions and experimental testing. J Mol Biol 2008;377(3):914-34.
36. Zhang M, Wang G, Shapiro A, Zhang JT. Topological folding and proteolysis profile of P-glycoprotein in membranes of multidrug-resistant cells: implications for the drug-transport mechanism. Biochemistry 1996;35(30):9728-36.
37. Li Z, Dong Z, Myer D, Yip-Schneider M, Liu J, Cui P, et al. Role of 14-3-3sigma in poor prognosis and in radiation and drug resistance of human pancreatic cancers. BMC Cancer 2010;10:598.
38. Qi J, Dong Z, Liu J, Zhang JT. EIF3i promotes colon oncogenesis by regulating COX-2 protein synthesis and beta-catenin activation. Oncogene 2014;33(32):4156-63.
39. Guvenc H, Pavlyukov MS, Joshi K, Kurt H, Banasavadi-Siddegowda YK, Mao P, et al. Impairment of glioma stem cell survival and growth by a novel inhibitor for Survivin-Ran protein complex. Clin Cancer Res 2013;19(3):631-42.
40. Peng H, Dong Z, Qi J, Yang Y, Liu Y, Li Z, et al. A Novel Two Mode-Acting Inhibitor of ABCG2-Mediated Multidrug Transport and Resistance in Cancer Chemotherapy. PLoS One 2009;4(5):e5676.
41. Engelsma D, Rodriguez JA, Fish A, Giaccone G, Fornerod M. Homodimerization antagonizes nuclear export of survivin. Traffic 2007;8(11):1495-502.
42. Chiou SK, Mandayam S. NSAIDs enhance proteasomic degradation of survivin, a mechanism of gastric epithelial cell injury and apoptosis. Biochem Pharmacol 2007;74(10):1485-95.
43. Chowdhury S, Howell GM, Teggart CA, Chowdhury A, Person JJ, Bowers DM, et al. Histone deacetylase inhibitor belinostat represses survivin expression through reactivation of transforming growth factor beta (TGFbeta) receptor II leading to cancer cell death. J Biol Chem 2011;286(35):30937-48.
44. Fortugno P, Beltrami E, Plescia J, Fontana J, Pradhan D, Marchisio PC, et al. Regulation of survivin function by Hsp90. Proc Natl Acad Sci U S A 2003;100(24):13791-6.
45. Giodini A, Kallio MJ, Wall NR, Gorbsky GJ, Tognin S, Marchisio PC, et al. Regulation of microtubule stability and mitotic progression by survivin. Cancer Res 2002;62(9):2462-7.
46. Tran J, Master Z, Yu JL, Rak J, Dumont DJ, Kerbel RS. A role for survivin in chemoresistance of endothelial cells mediated by VEGF. Proc Natl Acad Sci U S A 2002;99(7):4349-54.
47. Cheung CH, Chen HH, Kuo CC, Chang CY, Coumar MS, Hsieh HP, et al. Survivin counteracts the therapeutic effect of microtubule de-stabilizers by stabilizing tubulin polymers. Mol Cancer 2009;8:43.
48. Rosa J, Canovas P, Islam A, Altieri DC, Doxsey SJ. Survivin modulates microtubule dynamics and nucleation throughout the cell cycle. Mol Biol Cell 2006;17(3):1483-93.
49. Lens SM, Vader G, Medema RH. The case for Survivin as mitotic regulator. Curr Opin Cell Biol 2006;18(6):616-22.
50. Kitagawa M, Lee SH. The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis. Front Cell Dev Biol 2015;3:14.

Figure Legend

Figure 1. A. Overall structure of survivin dimer**.** The two subunits shown in ribbon are colored in cyan and orange, respectively. Interfacial non-core residues shown in stick representation are colored green. The deeply buried dimerization core is represented by their molecular surface in gray. **B.** Water molecules exchanged in the dimerization core unit from MD simulation.

Figure 2. Identification and characterization of LQZ-7. A. Non-denaturing PAGE analysis of compound effect on survivin dimerization. Purified survivin was incubated with different compounds followed by non-denaturing PAGE analysis of the protein and commassie blue staining. **B.** Chemical structure of LQZ-7. **C-D.** Dose-dependent effect of LQZ-7 on dimeric survivin (C) and 14-3-3σ (D) as described in panel A. **E.** Non-denaturing PAGE analysis of nascent survivin. Survivin cRNA was used to program cell-free translation in rabbit reticulocyte lysate in the absence or presence of LQZ-7. [^{35}S]-labeled nascent proteins were subjected to non-denaturing PAGE and autoradiography analysis. **F.** Dose-dependent binding of LQZ-7 to survivin. The intrinsic fluorescence of LQZ-7 was measured in the absence or presence of survivin.

Figure 3. LQZ-7 induces proteasome-dependent survivin degradation. A-C. Effect of LQZ-7 on the expression of endogenous and ectopic survivin. DU145 cells were treated with LQZ-7 or DMSO vehicle control for different times followed by Western blot analysis of survivin (A) or for 48 hrs followed by real-time RT-PCR analysis of survivin mRNA (B). Panel C shows Western blot analysis of LQZ-7 effect on ectopic Flag-tagged survivin (F-survivin) in HEK293 cells. **D-E.** Effect of LQZ-7 on survivin stability and half-life. DU145 and PC-3 cells were pre-treated with cycloheximide (CHX) to inhibit protein synthesis followed by chasing for different times in the presence of LQZ-7 or DMSO control and Western blot analysis of remaining survivin.

Panel E shows quantitation of survivin in panel D. **F**. Effect of proteasome inhibitors MG132 and bortezomib on LQZ-7-induced survivin degradation.

Figure 4. Characterization of LQZ-7 analogues. A. Chemical structures of LQZ-7 and its analogues. **B.** Effect of LQZ-7 and its analogues on PC-3 and DU145 cell survival as determined using MTT assay. **C.** Effect of LQZ-7 and its analogues on ectopic Flag-tagged survivin in HEK293 cells. **D.** Effect of LQZ-7F on endogenous survivin level in DU145 and PC-3 cells. **E.** Effect of LQZ-7F on survivin stability determined using cycloheximide (CHX) chasing as described in Fig. 3. **F.** Effect of proteasome inhibitors MG132 and bortezomib on LQZ-7F-induced survivin degradation. **G.** Pull-down assay. LQZ-7F was immobilized onto CNBr-activated Sepharose and used to pull down recombinant survivin, followed by separation on SDS-PAGE and silver staining. **H.** Predicted binding mode of LQZ-7F (stick and ball) in survivin (ribbon) using DOCK.

Figure 5. Effect of LQZ-7F on cancer cell survival. A. IC_{50} of LQZ-7F in human cell lines of different cancers as determined using MTT assay. 231, MDA-MB-231. **B.** Effect of LQZ-7F on human cancer cell survival as determined using colony formation assay. **C-D.** Apoptosis assay. Cells were treated without or with different concentrations of LQZ-7F for 24 hrs followed by AnnexinV/PI dual staining and analysis of apoptotic cells using flow cytometry (C) or subjected to Western blot analysis of cleaved PARP (D). **E.** Western blot analysis of survivin protein in different human cancer cell lines using 10 μg proteins of total cell lysate each. **F.** Scatter plot analysis of IC_{50} and relative survivin protein level of different cancer cell lines derived from 6 experiments.

Figure 6. Effect of LQZ-7F on microtubule structure. PC3 cells were treated without or with 2 μM LQZ-7F for 24 hrs followed by immunostaining of α-tubulin and counter stained with

DAPI. The images were captured using confocal microscopy.

Figure 7. In vivo efficacy of LQZ-7F. A-B. Effect of LQZ-7F on ectopic xenograft tumor growth and body weight in male NSG mice. Arrow heads indicate treatments. **C.** Gross anatomy of xenograft tumors. **D.** Final wet weight of xenograft tumors. (*$p<0.05$). **E-F.** Immunohistochemistry and Western blot analyses of survivin in xenograft tumors. Arrowheads indicate apoptotic cells with condensed chromatin.